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Duffy blood group phenotypes/genotypes and their association with malaria prevalence in four communities of northwest Ecuador.

Disertación previa a la obtención del título de Licenciada en Ciencias Biológicas

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Yo, Fabián Sáenz Director de la Disertación, certifico que la señorita Elizabeth Verónica Veloz Haro ha realizado la investigación sobre el tema: "Duffy blood group phenotypes/genotypes and their association with malaria prevalence in four communities of northwest Ecuador" de acuerdo a las normas y técnicas establecidas. Una vez concluido y revisado el trabajo, conforme con las disposiciones reglamentarias, autorizo la presentación del informe respectivo.

Quito, 26 de noviembre de 2015

Dr. Fabián Sáenz

Director de la disertación

A mi familia, en especial a mis padres

quienes me han brindado su apoyo incondicional…

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Duffy Blood Group Phenotypes/Genotypes and their Association with Malaria Prevalence in Four Communities of Northwest Ecuador.

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Abstract

Background

Ecuador is in malaria pre-elimination phase, with a reduction of more than 99% in the incidence of confirmed cases that were reported between 2000 and 2013. In Ecuador malaria is caused by *Plasmodium vivax* and *P. falciparum* protozoan species. Invasion of erythrocytes is essential for the development of the disease in the human host. *P. vivax* requires a specific interaction with antigens Fy^a or Fy^b, corresponding to the Duffy receptor in the red blood cells. The Duffy polymorphism affects *P. vivax* ability to invade

erythrocytes. This study aimed to determine the malaria prevalence and the composition of Duffy polymorphisms of the population of four communities of San Lorenzo county in northwest Ecuador.

Methodology/Findings

Blood samples from a total of 797 individuals were collected in El Pedregal, Ricaurte, La Boca and El Guadual communities in northwest Ecuador. *Plasmodium* detection and Duffy allele genotyping were performed by qPCR. The malaria prevalence in 2015 was compared to previously report in 2013. We found 4.5% malaria prevalence in 2015 that was lower than the reported prevalence for 2013. Most of positive infections (77.8%) were caused by *P. vivax* and 97.3% were asymptomatic. San Lorenzo population had 70.4% of Duffy positive phenotypes prevalence and 29.6% of Duffy negative phenotype prevalence. Six cases of *P. vivax* infected individuals with a Duffy negative genotype were found.

Conclusions

The results show a high prevalence of asymptomatic malaria in Northwest Ecuador being *P. vivax* the most frequent species detected. Moreover, the Duffy blood group system in the study sites is highly heterogeneous, with three alleles *FY*A*, $FY^{*}B$ and $FY^{*}B^{ES}$ present. In addition, we report for the first time in the pacific region of South America Duffy negative individuals infected with *P. vivax*. Taken together, the results show that the situation of malaria in northwest Ecuador is far from understood.

Author Summary

Malaria is a disease caused by *Plasmodium* protozoans and transmitted by Anopheles mosquitoes, that kills almost 584000 persons a year. Ecuador is a country of Northwest South America that has been very successful in controlling malaria and is now in pre-elimination phase. Nevertheless, several cases and outbreaks are still being reported.

Malaria cases are restricted to the Coast and Amazon regions of Ecuador, and is caused by two species, *P. vivax* and *P. falciparum.* Recent reports show a high asymptomatic prevalence than sustain transmission preventing malaria control and elimination. The genetic composition of the human population is an important factor that can influence the transmission process of malaria disease. In particular, the Duffy blood group polymorphisms affect *P. vivax* infection rates. The results of this study showed a high prevalence of asymptomatic malaria caused by *P. falciparum* and *P. vivax* with low parasitemias. Also it was found that in the study region, *P. vivax* is able to invade the red blood cells in a Duffy independent way.

Introduction

Malaria is a disease of tropical and subtropical countries caused by *Plasmodium* protozoans. Five species are recognized as natural malaria parasites of humans (*P. malariae, P. ovale, P. falciparum, P. vivax* and *P. knowlesi*). Due to its biology, *P. falciparum* is the specie that causes most mortality and *P. vivax* is the most widely distributed human malaria parasite in the world outside sub-Saharan regions of Africa [1]. Several studies have shown that in endemic malaria areas asymptomatic and submicroscopic infections are common [2-4]. In areas pursuing malaria elimination it is necessary to monitor all *Plasmodium* infections that could contribute to maintain transmission and persistence of malaria within endemic regions (including submicroscopic and asymptomatic infections that generally remain undetected and untreated) [3,4].

Ecuador is in malaria pre-elimination phase with a reduction of more than 99% in the incidence of confirmed malaria cases reported between 2000 and 2013. In 2000, 104600 cases were reported; in 2005, 17050 cases; in 2010, 1888 cases; in 2011, 1233 cases; in 2012, 558 cases; in 2013, 378 cases and in 2014, 243 cases [1,5,6]. In Ecuador the

presence of malaria is restricted to the Coast and Amazon regions, and is caused by two species of *Plasmodium: P. vivax* (that accounted 86% of the registered cases in 2012 and 83% in 2014) and *P. falciparum* (that caused 14% of the cases in 2012 and 17% in 2014) [1,7].

Invasion of erythrocytes is essential for the completion of the *Plasmodium* life cycle and development of malaria. *Plasmodium* invades and multiplies within host erythrocytes during the blood-stages of its life cycle [8]. The invasion process is complex and involves multiple steps. In particular, a specific binding and junction formation between the parasite ligands and erythrocyte receptors is essential for *Plasmodium* invasion of erythrocytes. This process varies greatly among different species of *Plasmodium: P. falciparum* uses multiple invasion pathways and multiple receptors while *P. vivax* requires interaction of the Duffy Binding Protein (DBP) with the antigens Fy^a or Fy^b, from a single receptor called Duffy or DARC (Duffy antigen receptor for chemokines) [9-12]. Therefore, Duffy polymorphisms are implied in the susceptibility or resistance of human populations to *P. vivax* [10,11,13,14].

The Duffy polymorphisms are the result of the different combinations of the three main alleles of the *Fy* gene: *FY*A*, *FY*B* and $FY*B^{ES}$ (ES: stands for erythrocyte silent). *FY*A* and *FY*B* alleles express the antigens Fy^a or Fy^b respectively; the SNP that differentiates this alleles is a single nucleotide substitution at position 125 (G>A) [15,16]. The *FY*A, FY*B* alleles expression is codominant and results in four possible phenotypes: Fy(a+b-), Fy(a-b+), Fy(a+b+) and Fy(a-b-) [16,17]. The first three correspond to Duffy positive phenotypes. The Fy(a+b-) phenotype predominates in southeast Asia and in many American populations, the Fy(a-b+) phenotype predominates in European populations, the Fy(a+b+) predominates in North Africa and some American populations [11].

The negative phenotype Fy(a-b-) is associated with a single nucleotide mutation in the GATA-box promoter region of the *FY* gene, (C>T) at position -33, which disrupts a binding site for the GATA-1 erythroid transcription factor. In fact, it abolishes the receptor´s expression [11,13,18]. The absence of the Duffy receptor confers resistance to *P. vivax* infection [12-14,16,18,19]. This phenotype predominates in some populations of Africa where malaria is endemic, but *P. vivax* reported cases are low or non-existent [11,17,19]. Several new reports show infection of *P. vivax* in Duffy negative individuals in Kenya [20], Ethiopia [21], Madagascar [22], Mauritania [23], Brazilian Amazon [24,25], Angola and Equatorial Guinea [26], suggesting that *P. vivax* may also have redundancy in invasion pathways resulting from a recent evolution of the parasite.

The Duffy blood group distribution and the incidence of malaria in Rio Cayapas region (Esmeraldas province) northwest Ecuador was studied in 1985. The population was composed of two distinct racial groups: Blacks (African descent) and Chachi (indigenous population). The results showed an incidence twice higher of *P. vivax* infection in the Chachi group than what was found in the Black population, this last one with 76% prevalence of Duffy negative phenotype [27]. The present study aimed to determine the malaria prevalence and Duffy genotypes in four malaria-endemic locations in San Lorenzo county of Esmeraldas province using quantitative PCR (qPCR) and identify a possible association between the two in order to improve our understanding of the malaria situation in the area.

Methods

Ethics statement and surveys

The ethical and methodological aspects of this study were carried out in accordance with international ethics procedures for the use of human subjects in research and were

approved by Pontificia Universidad Católica del Ecuador Ethics Committee. Each participant was informed of the aims of the study and the volunteers signed a written informed consent.

A symptoms survey was answered by the participants. The ethnicity data was obtained through a knowledge, attitude and practices (KAPs) survey.

Study area and population

The samples for this study were collected in February 2015 in a cross-sectional study conducted in Ecuadorian malaria endemic areas: four communities (El Pedregal, Ricaurte, La Boca and El Guadual) were selected in Esmeraldas province, San Lorenzo county in northwest Ecuador. El Pedregal is located in San Lorenzo city and has 270 inhabitants. Ricaurte is located about 15 km southeast from San Lorenzo city, with a population of 1381 inhabitants. La Boca is located 7 km southwest of Ricaurte with approximately 580 inhabitants. El Guadual is located about 40 km southeast from La Boca and it has a population of approximately 280 inhabitants (Fig. 1). El Pedregal, Ricaurte and La Boca are lowland communities located at 30 masl, 41 masl and 30 masl respectively, with predominant Afro-descendants population. While El Guadual is a community located at 649 masl with predominant Mestizo population.

Blood samples and DNA extraction

The sample size was calculated for each community with a confidence level of 95%, using OpenEpi v.3.03a software. In total 797 samples were examined (El Pedregal, n=151, Ricaurte, n=281, La Boca n= 226 and El Guadual n= 139). Blood samples were collected on filter paper (Whatman 3MM chromatography paper) and DNA was extracted from dried blood spots using the PureLink Genomic DNA mini kit (Invitrogen).

Parasite detection and comparison with malaria prevalence in 2013

The molecular diagnosis of malaria infection and identification of *Plasmodium* species was performed using real time PCR, Applied Biosystems 7500 v.2.0.5 analytical PCR system. The amplification was done using primers that target the cytochrome oxidase 1 (CO1) of *P. vivax* or *P. falciparum*, following the protocol of CLAIM (Centro Latinoamericano de Investigación en Malaria). The qPCR reactions were carried out in 96-well 0.1 ml thin-wall optical PCR plates with optical adhesive film sealing tapes (Applied Biosystems). The 10 µl reaction mixture contained 2 µl of genomic DNA, 5 µl of TaqMan Universal PCR Master Mix (Applied Biosystems), 10 µM of each parasite species-specific primer, 10 µM of each fluorescence labelled probes to hybridize differentially to *P. vivax* or *P. falciparum* enabling species identification. The primer and probe sequences are listed in Table 1. The PCR amplification profile used was previously described with minor modifications [28-30], and consisted of an initial denaturation at 95°C for 10 min, 45 cycles of 95°C for 15s and 60° for 1 min. Standard *P. falciparum* and *P. vivax* DNA positive and negative controls were used. The parasitemia quantification was performed using a parasite-specific standard curve obtained by diluting controls.

Malaria prevalence, asymptomatic infections and *Plasmodium* species prevalence were compared with the results of a previous study conducted in 2013 in the same communities [31]. The formula "$\Delta_{\%}= \frac{data\ 2015 - data\ 2013}{data\ 2013}$" was used to calculate the variation percentage of each variable in two years.

Duffy blood group genotyping

Forty percent of samples of each community were randomly selected (all the samples infected with *P. vivax* and *P. falciparum* were included in the analysis). The Duffy allele genotyping was performed by qPCR as described by Sousa *et al.* [32]. A 10 µl PCR mixture was performed using 2 µl of genomic DNA, 5 µl of SYBR Green PCR Master

Mix (Applied Biosystems), four different combinations of primer pairs (adapted from [32]). The primer sequences are listed in Table 1. In each plate, positive controls were included for genotypes FY*A/FY*A, FY*A/FY*B, FY*A/FY*B^{ES}, and FY*B^{ES}/FY*B^{ES}. The amplification and fluorescence detection were performed using an Applied Biosystems 7500 v.2.0.5 analytical PCR system under the following conditions: initial denaturation at 95°C for 10 min, then 35 cycles of 95°C for 15 s and 60°C for 1 min.

Comparison of Duffy phenotypes/genotypes in lowland communities and El Guadual and among *P. vivax*-infected and non *P. vivax*-infected individuals

The data obtained of phenotypes, genotypes and allelic prevalences of Duffy blood group were compared between lowland communities (El Pedregal, Ricaurte and La Boca) and El Guadual (located in a higher area of San Lorenzo county).

The data obtained of phenotypes, genotypes and allelic prevalences of Duffy blood group were compared among *P. vivax* infected, *P. falciparum* infected and non-infected individuals. *P. falciparum* infected and non-infected individuals were analyzed together as a control group because *P. falciparum* does not use Duffy antigens to invade erythrocytes.

Statistical analysis

SPSS 18 was used for statistical analysis of the data. Chi-square test (χ^2) was used to compare the malaria prevalence, asymptomatic infections and *Plasmodium* species prevalence between 2013 and 2015. Chi-square test (χ^2) and Fisher´s exact test were used to compare the proportions of Duffy phenotypes, genotypes and alleles among communities (lowland communities and El Guadual) and among the infections (*P. vivax*-infected and non *P. vivax*-infected individuals).

Results

Ethnic composition of the study communities

El Pedregal, Ricaurte and La Boca are predominantly Afro-Ecuadorian communities: At the community of El Pedregal, 80% of respondents identified themselves as Afro-Ecuadorians, 12.5% as Mestizo and 7.5% as White-Caucasian. At Ricaurte and La Boca more than 90% respondents identified themselves as Afro-Ecuadorians and the remaining as Mestizos. At El Guadual 67% of the surveyed identified themselves as Mestizos and the remaining as Afro-Ecuadorians (Fig. 2).

Parasite Detection

The overall malaria prevalence by qPCR in the four studied communities of San Lorenzo county was 4.5% (36/797). The prevalence of infection was much higher in El Guadual (10.8%), followed by La Boca (4.0%), Ricaurte (2.8%) and El Pedregal (2.6%) (Table 2). The two main species of *Plasmodium* were identified. *P. falciparum* showing the lowest infection rates (0.7% in El Pedregal and Ricaurte, 0.9% in La Boca and 2.2% in El Guadual) and *P. vivax* with the highest infection rates in all communities. *P. vivax* was detected in 28 individuals, 3 from El Pedregal (2%), 6 from Ricaurte (2.1%), 7 from La Boca (3.1%) and 12 from El Guadual (8.6%) (Fig. 3A). The overall parasite distribution was 77.8% for *P. vivax* and 22.2% for *P. falciparum* (Fig. 3B). No mixed infections were detected.

The mean parasitemia (parasites/µl) determined by qPCR in El Pedregal was 2.25 (1-6 parasites/µl), in Ricaurte it was 23.88 (1-144 parasites/µl), in La Boca it was 7.78 (1-23 parasites/µl) and in El Guadual it was 74.67 (1-590 parasites/µl) (Fig. 3C). Among the positive cases only 1 presented symptoms with 10 parasites/µl of parasitemia, resulting in 97% of asymptomatic infections (Fig. 3D). The prevalence of malaria infections in the blood samples is presented in Table 2.

Table 3 shows a comparison with a study conducted in 2013 in the same communities [31]. We observed 39.2% decrease of malaria prevalence in the study area (7.4% in 2013 to 4.5% in 2015), which was significant (p=0.013). Nevertheless in Ricaurte and El Guadual communities the malaria prevalence increased in 64.7% and 96.4% respectively, even though the increase was not significant (p=0.299 and p=0.150) (Fig. 4A). A reduction of *P. vivax* prevalence (50%) and an increase of *P. falciparum* prevalence (100%) were observed (Fig. 4B), but only the reduction of *P. vivax* prevalence was significant (p=0.002). The asymptomatic infection percentage was similar in 2013 and 2015 (98% and 97%, respectively).

Duffy blood group genotyping

The data shows 70.4% of Duffy positive phenotypes. Out of these, the Fy(a+b-) was the predominant phenotype with a 57.6% prevalence (*FY*A/FY*B*^{ES} was the most frequent genotype, followed by *FY*A/FY*A*). The percentages for Fy(a-b+) and Fy(a+b+) phenotypes were low (8.5% and 4.4%, respectively). The negative Duffy phenotype Fy(a-b-) (with genotype *FY*B*^{ES}*/FY*B*^{ES}) showed a 29.6% prevalence. The lowest allelic frequency was 0.079 for the *FY*B* allele whereas *FY*A* and *FY*B*^{ES} alleles had similar frequencies (0.436 and 0.486, respectively). The phenotypic, genotypic and allelic frequencies of the Duffy blood group in the 319 analyzed samples are summarized in Table 4. Representative graphs of melting curves for each genotype are presented in Figure 5.

The Duffy positive phenotypes were the most common in the four communities of San Lorenzo (Fig. 6A), with a significant difference (p=0.000) when comparing the lowland communities and El Guadual. We observed an association between the Duffy positive phenotype with El Guadual (91.1%) and an association between the Duffy negative phenotype and lowland communities (34%) (Table 5).

The genotype distribution varied among the study sites. In El Pedregal the most common genotype was *FY*A/FY*A* with 33.3% and the lowest was *FY*B/FY*B* with 3.3%. In Ricaurte the most common genotype was $FY*B^{ES}/FY*B^{ES}$ with 39% and the lowest *FY*A/FY*B* with 0.9%. In La Boca the most common genotype was $FY*A/FY*B^{ES}$ with 48.9% and the lowest $FY*B/FY*B^{ES}$ with 2.2%. In El Guadual the most common genotype was *FY*A/FY*A* with 53.6% and the lowest $FY*B/FY*B^{ES}$ with 3.6%, while the *FY*B/FY*B* genotype was absent (Fig. 6B).

When comparing the genotypes between lowland communities and El Guadual we observed significant differences with *FY*A/FY*A*, *FY*A/FY*B* and $FY*B^{ES}/FY*B^{ES}$ genotypes (p=0.000, p=0.022 and p=0.000, respectively). We observed an association between lowland communities with the $FY*B^{ES}/FY*B^{ES}$ genotype, and association of El Guadual with *FY*A/FY*A* and *FY*A/FY*B* genotypes (Table 5). Additionally, when we compared the allele prevalence, an association between $FY*B^{ES}$ allele with lowland communities and *FY*A* allele with El Guadual were found (p=0.000) (Table 5).

Duffy blood group genotypes in *P. vivax*-infected and non *P. vivax*-infected individuals

Table 6 shows a comparison of the results of inferred Duffy phenotypes among *P. vivax* infected individuals and a control group (*P. falciparum* infected and non-infected individuals). In the control group 69.7% were Duffy positives and 30.3% were Duffy negative. Among *P. vivax* positive by qPCR, 78.6% were Duffy positives and 21.4% were Duffy negative (six individuals). Of these six Duffy negative individuals one was from El Pedregal, four from Ricaurte and one from La Boca. The risk of Duffy negatives to experience a *P. vivax* blood stage infection was lower but not significantly different from that of Duffy positives (p=0.224).

No significant differences were observed when comparing the genotypic prevalence of the Duffy blood group among *P. vivax* infected and control group in El Pedregal, Ricaurte and La Boca communities. However, in El Guadual the *FY*A/FY*B*^{ES} genotype showed association with the resistance to *P. vivax* infection (p=0.027). When all samples were analyzed together, we observed an association between the *FY*A/FY*A* genotype and the risk of *P. vivax* infection (p=0.025) (Table 7).

With respect to the *FY* alleles frequency, there was a significant difference between *P. vivax* infected and the control group only for *FY*B*^{ES} allele (p=0.024 and p=0.033 in El Guadual and total samples, respectively). There was a 0.498 *FY*B*^{ES} allele frequency in the control group and 0.357 *FY*B*^{ES} allele frequency in *P. vivax* infected individuals, showing an association between *FY*B*^{ES} allele and the resistance to *P. vivax* infection (Table 8).

Discussion

The prevalence of *Plasmodium* and Duffy blood group phenotypes and genotypes was studied in four communities of San Lorenzo, Esmeraldas province, an area of malaria transmission in the north Ecuadorian Coast, in order to analyze the relation between *P. vivax* infection and Duffy blood group polymorphisms.

This study shows a 4.5% malaria prevalence out of which 97% were asymptomatic. *P. vivax* was the most common species detected (3.5%) and only 1% prevalence of *P. falciparum* was identified (Fig. 4). When comparing these results to the study conducted in the same communities by Sáenz *et al*. in 2013 [31], we observed a significant decrease of 39.2% in malaria prevalence (7.4% in 2013, 4.5% in 2015). Nevertheless, Ricaurte and El Guadual showed an increase in malaria prevalence which was not significant (Fig. 5A). In 2013, 94% of *Plasmodium* infections were caused by *P. vivax* and 6% were caused by *P. falciparum*; while in 2015, 78% of *Plasmodium* infections were caused by *P. vivax* and

22% were caused by *P. falciparum.* The data show a significant reduction of *P. vivax* prevalence and no significant increase of *P. falciparum* prevalence in the study site (p=0.002 and p=0.194, respectively) (Fig. 5B).

The positive cases in the present study have low parasitemias (1-590 parasites/µl; mean: 27.22 parasites/µl) according to the parasitemia classification described by Bamaga *et al*. in 2014 [33]. These results are consistent with previous studies in malaria endemic areas where *P. vivax* is the predominant species and the prevalence of sub-microscopic infections varies between 3 to 20% [2,30]. In South America a high prevalence of sub-microscopic and asymptomatic infections were reported (between 59% and 98%), in countries such as Colombia [30], Ecuador [31], Perú [34], Venezuela and Brazil [35,36].

The asymptomatic cases can act as reservoirs of parasites and asymptomatic carriers can transmit the parasite to other individuals preventing malaria control and elimination [3,4,30]. The results obtained in this study are relevant because they show high asymptomatic and sub-microscopic malaria prevalence in the northwest of the country. In particular, in El Guadual, the community with higher prevalence, there was a *P. falciparum* outbreak (19 clinical cases) [6] shortly after sampling, that could have been originated by asymptomatic infections identified in this study. The molecular diagnosis is important to identify low parasitemias, frequently asymptomatic, which are not detected by standard methods but can be transmitted and cause clinical outbreaks [30,37]. Consequently, asymptomatic infections should be taken in account in elimination policies of the disease in the region [37,38].

Although the Duffy blood group distribution and the incidence of malaria in Rio Cayapas in Esmeraldas were studied in 1985, little is known on the frequency of red blood cell polymorphism that confers either partial or complete resistance against malaria in Ecuador. In the previous study, 62.5% of Duffy positive and 37.5% of Duffy negative

phenotypes were found in lowland communities, and the incidence of *P. vivax* was twice high in the Duffy positive individuals than in the Duffy negative subjects [27]. El Pedregal, Ricaurte and La Boca are lowland communities of San Lorenzo county and the percentages of Duffy phenotypes are similar to what was found by Guderian *et al.* in Rio Cayapas, with 67% of Duffy positive and 33% of Duffy negative phenotype. The variation found is possibly due to population changes that have occurred over time, such as new population mixtures and migrations. On the other hand, El Guadual community is located in a higher area of San Lorenzo county (649 masl), and 91% of Duffy positives and 9% of Duffy negatives phenotypes were found. These results can be explained because of the ethnic composition of this community, with predominant Mestizo population (66,7%) in opposition to Afro-descendants in the lowland communities (El Pedregal, Ricaurte and La Boca).

The Duffy blood group system in San Lorenzo communities is polymorphic with three alleles *FY*A*, *FY*B* and $FY*B^{ES}$ presented; the data obtained demonstrates high heterogeneity of these alleles in the population. When analyzing the allele frequencies, the most common allele was $FY*B^{ES}$ (0.486), although there was a higher prevalence of Duffy positive phenotypes, because of the great amount of heterozygous genotype ($FY*A/FY*B^{ES}$). As it has been previously reported [27], in this study we found an association between the Duffy positive phenotype with Mestizo population and an association between Duffy negatives and Afro-Ecuadorians population (p=0.000). Also we observed an association between lowland communities with $FY*B^{ES}/FY*B^{ES}$ genotype and $FY*B^{ES}$ allele, and an association of El Guadual with *FY*A/FY*A* and *FY*A/FY*B* genotypes, and the *FY*A* allele (p-values in Table 5). These results are consistent with previous reports that show a relationship between the ethnic composition and the different

Duffy phenotypes, mainly African descendents with Duffy negative phenotype [13,16,18,19,27].

When comparing the phenotype prevalence between *P. vivax* infected individuals and the control group (*P. falciparum* infected and non-infected individuals) no significant differences were observed, emphasizing the importance of the evaluation of Duffy polymorphism in malaria endemic areas. Although it is know that Duffy negative genotype confers resistance to *P. vivax* blood-stage infection, in the current study, six *P. vivax* infected individuals that presented a Duffy negative genotype were reported: one in El Pedregal, four in Ricaurte and one in La Boca. Therefore, the risk of Duffy negatives to experience a *P. vivax* blood stage infection was lower but not significantly different to that of Duffy positives ($p=0.224$). Similar results were found in other studies with recent reports of different parts of malaria endemic area, such as Kenya [20], Ethiopia [21], Madagascar [22], Mauritania [23] and the Brazilian Amazon [24,25]. Nevertheless, this is the first report of Duffy negative individuals infected with *P. vivax* in the Pacific region of South America.

The reason by which Duffy negative individuals could be infected by *P. vivax* has not been determined. Several factors may be involved, mainly the species biology because of its extraordinary capacity to adapt [21,26]. In regions where the Duffy positive phenotype is most common, people could be a reservoir of *P. vivax* giving the opportunity to infect hepatocytes of Duffy negative people and the capacity to develop new forms to invade erythrocytes [21,22,24,26]. Another factor to take into account is that *P. vivax* could be able to infect human red cells through a Duffy independent mechanism; using more than a pathway of invasion, possibly by an alternative receptor [26]. In addition new *P. vivax* strains that do not depend of the Duffy antigen for invasion could be appearing [21].

We did not find significant differences in Duffy genotypes among *P. vivax* infected and control groups in El Pedregal, Ricaurte and La Boca communities. These results are inconsistent with previews reports than show a relationship between the genotypes and the susceptibility of *P. vivax* infection [10,11,13,14], but similar results were found in Anajás-Brazil, where Carvalho *et al.* did not find different frequencies between genotypes that had a higher prevalence of Duffy positive people [24]. However, in El Guadual the *FY*A/FY*B*^{ES} genotype showed an association with the resistance to *P. vivax* infection (p=0.027). When the total samples were analyzed together we observed an association between the *FY*A/FY*A* genotype and the *P. vivax* risk (p=0.025). This result is inconsistent with King *et al.* [11] who found that the Fy^a antigen is associated with protection because it reduces the risk of *P. vivax* in humans compared with Fy^b, in an in-vitro experiment. This apparent discrepancy may be due to the genetic composition of El Guadual community where the majority of the people have a *FY*A/FY*A* genotype. Also there are other possible risk factors that influence a high prevalence in this community such as the knowledge, attitudes and practices towards malaria this community [38] or possibly a greater presence of the vector.

The frequency of *FY*B*^{ES} was greater in the control group than in *P. vivax* infected individuals when the alleles were analyzed (p=0.024 in El Guadual and p=0.033 globally), suggesting that as presented elsewhere there is an association with the resistance to be infected by *P. vivax* and this allele. This is consistent with reports than manifest the presence of the *FY*B*^{ES} allele may be a selective advantage because reducing the rate of infection by *P. vivax* [11-14,16,30,31] and show that the presence of the *FY*B*^{ES} allele results in a 50% reduction in the Duffy protein expression on the erythrocyte surface when it is present in a heterozygote form [39,40].

In conclusion, the results obtained in this study are highly relevant. First, they confirm that the presence of sub-microscopic and asymptomatic malaria infections in Ecuador. Therefore, it is necessary to employ more sensitive techniques such as qPCR for correct diagnosis and treatment to eliminate malaria in endemic regions, together with policies, plans and programs that the Ministry of Public Health needs to implement.

Second, these findings show for the first time in the pacific of South America the ability of *P. vivax* to infect Duffy negative individuals. Although we do not know which are the circumstances that lead to this process, it is important to explore the different mechanisms of *P. vivax* infection and its ability to adapt, because it can result in one of the causes that difficult the elimination of this parasite.

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References

1. WHO (2015) World Malaria Report 2014: 1-242.

2. Moreira CM, Abo-Shehada M, Price RN, Drakeley CJ (2015) A systematic review of sub-microscopic *Plasmodium vivax* infection. Malaria Journal 14: 360.

3. Bousema T, Okell L, Felger I, Drakeley C (2014) Asymptomatic malaria infections: detectability, transmissibility and public health relevance. Nature Reviews Microbiology 1-8.

4. Laishram DD, Sutton PL, Nanda N, Sharma VL, Sobti RC, Carlton JM, Joshi H (2012) The complexities of malaria disease manifestations with a focus on asymptomatic malaria. Malaria Journal 11: 29.

5. Ministerio de Salud Pública del Ecuador "MSP" (2011) Situación de la malaria, Tendencia actual y sus desafíos para el control en el Ecuador.

6. National Service Control of vector-borne deseases "SNEM" (2015) Data reported by SNEM.

7. Vélez EC (2014) Caracterización poblacional de *Plasmodium vivax* en Ecuador mediante microsatélites y análisis de resistencia frente a antimaláricos.

8. Greenwood BM, Fidock DA, Kyle DE, Kappe SHI., Alonso PL, Collins FH, Duffy PE (2008) Malaria: progress, perils, and prospects for eradication. The Journal of Clinical Investigation 118: 1266-1276.

9. Persson KEM, McCallum FJ, Reiling L, Lister NA, Stubbs J, Cowman AF, Marsh K, Beeson JG (2008) Variation in use of erythrocyte invasion pathways by *Plasmodium falciparum* mediates evasion of human inhibitory antibodies. The Journal of Clinical Investigation 118:342-351.

10. Rayner JC, Vargas-Serrato E, Huber CS, Galinski MR, Barnwell JW (2001) A *Plasmodium falciparum* homologue of *Plasmodium vivax* reticulocyte binding protein

(PvRBP1) defines a trypsin-resistant erythrocyte invasion pathway. J Exp Med 194(11): 1571-1581.

11. King CL, Adams JH, Xianli J, Gimberg BT, McHenry AM, Greenberg LJ, Siddiqui A, Howes RE, da Silva-Nunes M, Ferreira MU, Zimmerman PA (2011) Fy^a/Fy^b antigen polymorphism in human erythrocyte Duffy antigen affects susceptibility to *Plasmodium vivax* malaria. PNAS 108: 20113-20118.

12. Grimberg BT, Udomsangpetch R, Xainli J, McHenry A, Panichakul T, Sattabongkot J (2007) *Plasmodium vivax* invasion of human erythrocytes inhibited by antibodies directed against the Duffy binding protein. PLoS Med 4(12): 337.

13. Miller LH (1994) Impact of malaria on genetic polymorphism and genetic diseases in Africans and African Americans. Proc Matl Acad Sci USA 91(7): 2415-2419.

14. Cavasini C, Mattos L, D´Almeida A, D´Almeida V, Gollino Y, Moretti L, Bonini C, Rossit A, Castilho L, Machado R (2007). Duffy blood group gene polymorphisms among malaria vivax patients in four areas of the Brazilian Amazon region. Malaria Journal 6: 167.

15. Iwamoto S, Omi T, Kajii E, Ikemoto S (1995) Genomic organization of the glycophorin D gene: Duffy blood group Fy^a/Fy^b alloantigen system is associated with a polymorphism at the 44-amino acid residue. Blood 85: 622-626.

16. Zimmerman P, Ferreira M, Howes R, Mercereau-Puijalon O (2013) Red Blood Cell Polymorphism and Susceptibility to *Plasmodium vivax*. Adv Parasitol 81: 27–76.

17. Castilho L, Ríos M, Pellegrino J, Saad S, Costa F, Reid M (2001) A novel FY allele in Brazilians. Vox Sanguinis 87:190-195.

18. Barbosa G (2011) Duffy Blood Group System and the malaria adaption process in humans. Rev Bras Hematol Hemoter 33(1): 55-64.

19. Hedrick PW (2011) Population genetics of malaria resistance in humans. Heredity 107: 283-304.

20. Ryan JR, Stoute JA, Amon J, Dunton RF, Mtalib R, Koros J, Owour B, Luckhart S, Wirtz RA, Barnewll JW, Rosenberg R (2006) Evidence for transmission of *Plasmodium vivax* among a Duffy antigen negative population in Western Kenya. Am J Trop Med Hyg 75(4): 575-581.

21. Woldearegai TG, Kremsner PG, Kun JFJ, Mordmüller B (2013) *Plasmodium vivax* malaria in Duffy-negative individuals from Ethiopia. Trop Med Hyg 107: 328-331.

22. Ménard D, Barnadas C, Bouchier C, Henry-Halldin C, Gray LR, Ratsimbasoa A, Thonier V, Carod JF, Domarle O, Colin Y, Bertrand O, Picot J, King CL, Grimberg BT, Mercereau-Puijalon O, Zimmerman PA (2010) Plasmodium vivax clinical malaria is commonly observed in Duffy-negative Malagasy people. PNAS 107:5967-5971.

23. Wurtz N, Lekweiry KM, Bogreau H, Pradines B, Rogier C, Boukhary AOMS, Hafid JE, Salem MSOA, Trape JF, Basco LK, Briolant S (2011) Vivax malaria in Mauritania includes infection of a Duffy-negative individual. Malaria Journal 10: 336.

24. Carvalho TA, Queiroz MG, Cardoso GL, Diniz IG, Silva AN, Pinto AY, Guerreiro JF (2012) *Plasmodium vivax* infection in Anajás, State of Pará: no differential resistance profile among Duffy-negative and Duffy-positive individuals. Malaria Jounal 11: 430.

25. Cavasini CE, de Mattos LC, D'Almeida AA, Bonini-Domingos CR, Herrera S, de Souza WC, Alves RT, Baptista AR, Castilho L, Dantas RL (2007) *Plasmodium vivax* infection among Duffy antigen-negative individuals from the Brazilian Amazon region: an exception?. Trans R Soc Trop Med Hyg 101: 1042-1044.

26. Mendes C, Dias F, Figueiredo J, Gonzalez V, Cano J, de Sousa B, E.do Rosáiro V, Benito A, Berzosa P, Ares AP (2011) Duffy negative antigen is no longer a barrier to

Plasmodium vivax – molecular evidences from the African West Coast (Angola and Equatorial Guinea). PLoS Negl Trop Dis 5: e1192.

27. Guderian R, Vargas J (1985) Duffy blood group distribution and the incidence of malaria in Ecuador. Trans R Soc Trop Med Hyg 80(1): 162-163

28. Herrera S, Vallejo AF, Quintero JP, Arévalo M, Cancino M, Ferro Santiago (2014) Field evaluation of an automated RDT reader and data management device for *Plasmodium falciparum/Plasmodium vivax* malaria in endemic areas of Colombia. Malaria Journal 13:87

29. Vallejo AF, Martínez NL, González IJ, Arévalo M, Herrera S (2015) Evaluation of the Loop Mediated Isothermal DNA Amplification (LAMP) Kit for Malaria Diagnosis in *P. vivax* Endemic Settings of Colombia. PLoS Negl Trop Dis 9: 3452

30. Vallejo AF, Chaparro PE, Benavides Y, Álvarez A, Quintero JP, Padilla J, Arévalo M, Sócrates H (2015) High prevalence of sub-microscopic infection in Colombia. Malaria Journal 14(201): 1-7

31. Sáenz FE, Arévalo A, Valenzuela G, Vallejo A, Castellanos A, Poveda A, Gutiérrez J, Álvarez A, Yang YH, Benavidez J, Castro E, Arévalo M, Herrera S. Malaria epidemiology in low endemicity areas of the northern coast of Ecuador. (On review).

32. Souza TN, Sanchez BAM, Cerávolo IP, Carvalho LH, Brito CFA (2007) Real-time multiplex allele-specific polymerase chain reaction for genotyping of the Duffy antigen, the *Plasmodium vivax* invasion receptor. Vox Sang 92: 373-380.

33. Bamaga O, Mahdy M, Mahmud R, Lim Y (2014) Malaria in Hadhramout, a southeast province of Yemen: risk factor, knowledge, attitude and practices (KAPs). Parasites and Vectors 7(351): 1-8

34. Branch O, Casapia WM, Gamboa DV, Hernandez JN, Alava FF, Roncal N, Álvarez E, Pérez EJ, Gotuzzo E (2005) Clustered local transmission and asymptomatic

Plasmodium falciparum and *Plasmodium vivax* malaria infections in a recently emerged, hypoendemic Peruvian Amazon community. Malaria Journal 4:27

35. Silva M, Moreno M, Conn JE, Gamboa D, Abeles S, Vinetz JM, Ferreira MU (2012) Amazonian malaria: Asymptomatic human reservoirs, diagnostic challenges, environmentally-driven changes in mosquito vector populations, and the mandate for sustainable control strategies. Acta Trop 121(3): 281-291

36. Arevalo M, Quiñones ML, Guerra C, Céspedes N, Giron S, Ahumada M, Piñeros JG, Padilla N, Terrientes Z, Rosas A, Padilla JC, Escalante AA, Beier JC, Herrera S (2012) Malaria in selected non-Amazonian countries of Latin America. Acta Trop 121(3): 303-314.

37. Herrera S, Ochoa SA, González IJ, Peinado L, Quiñones ML, Arévalo M (2015) Prospects for malaria elimination in Mesoamerica and Hispaniola. PLoS Negl Trop Dis 1-15.

38. Poveda AC (2015) Determinación de la prevalencia de Plasmodium spp. Mediante microscopía y su relación con conocimientos, actitudes y prácticas de malaria como posibles factores de riesgo en cuatro comunidades del cantón San Lorenzo provincia de Esmeraldas.

39. Michon P, Wooley I, Wood EM, Kastens W, Zimmerman PA, Adams JH (2001) Duffy-null promotor heterozygosity reduces DARC expression and abrogates ashesion of the *P. vivax* ligand required for blood-stage infection. FEBS Lett 495:111-114.

40. Yazdanbakhsh M, Rios M, Storry JR, Kosower N, Parasol N, Chaudhuri A, Reid ME (2000) Molecular mechanism that lead to reduce expression of Duffy antigens. Transf 40:310-320.

Figures

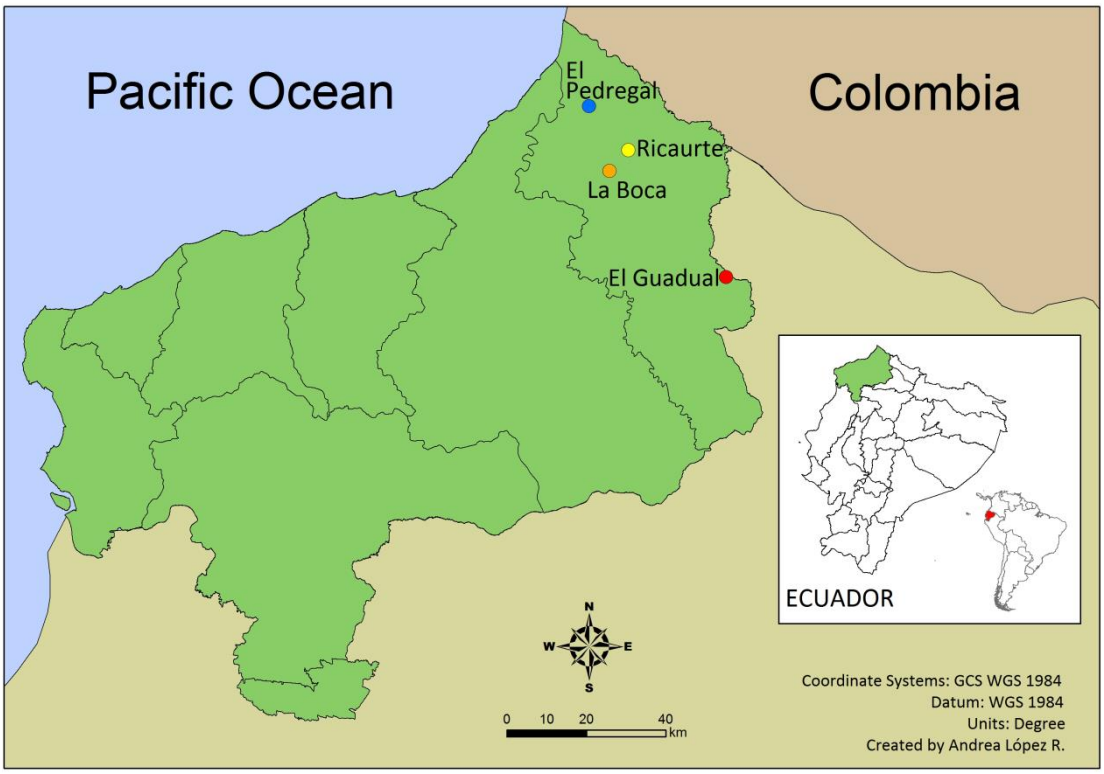


Figure 1. Study sites. Map of the four study sites in San Lorenzo county, Esmeraldas province in northwest Ecuador.

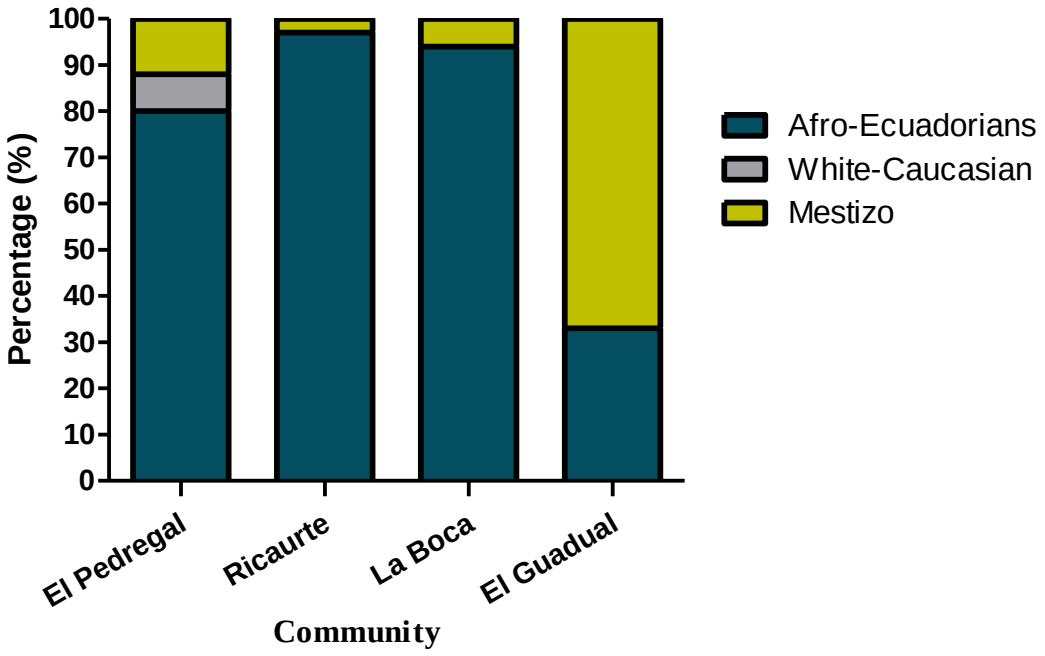
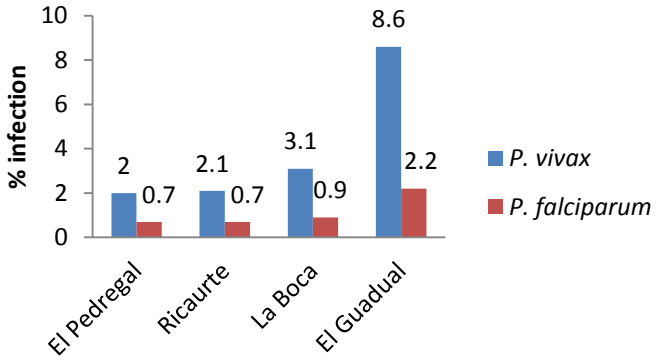
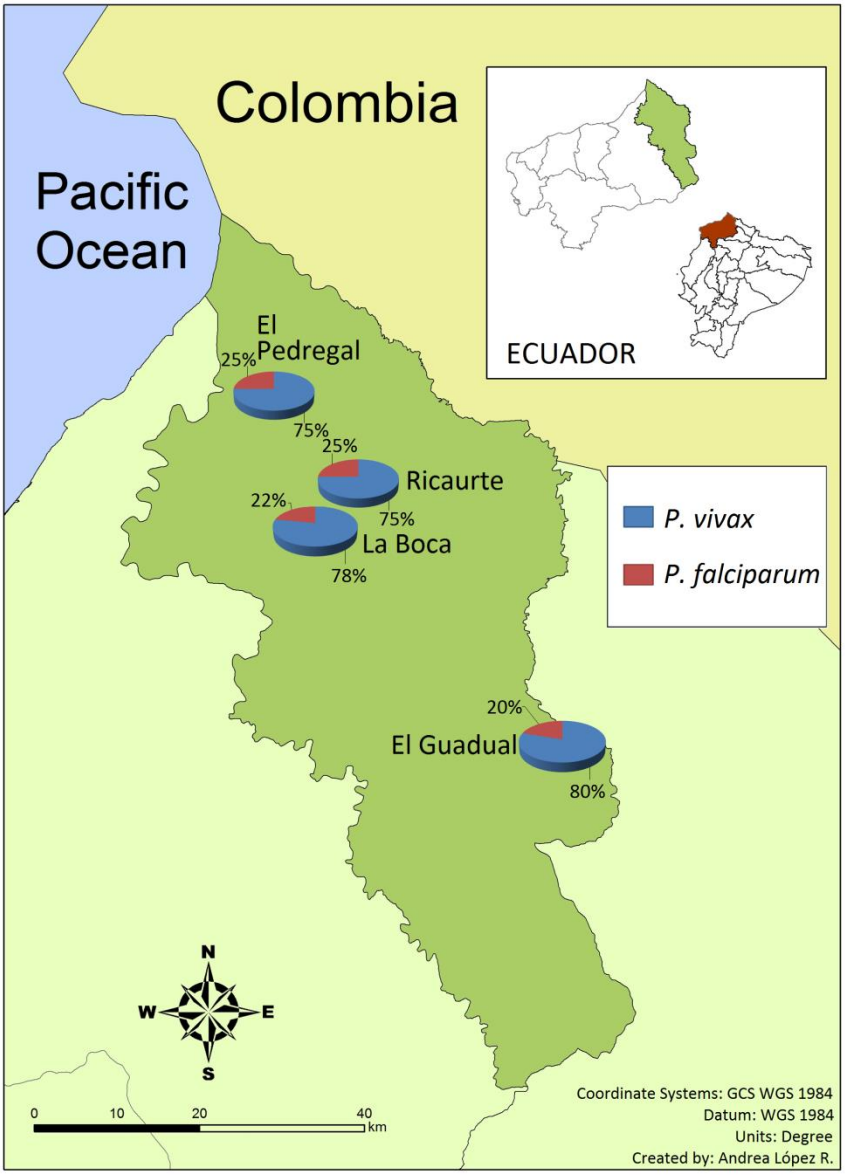


Figure 2. Ethnic composition of the study communities. El Pedregal, Ricaurte and La Boca communities are predominantly Afro-Ecuadorian and El Guadual community is predominantly Mestizo. A small portion of the population in El Pedregal community identified themselves as White-Caucasian (7.5%).

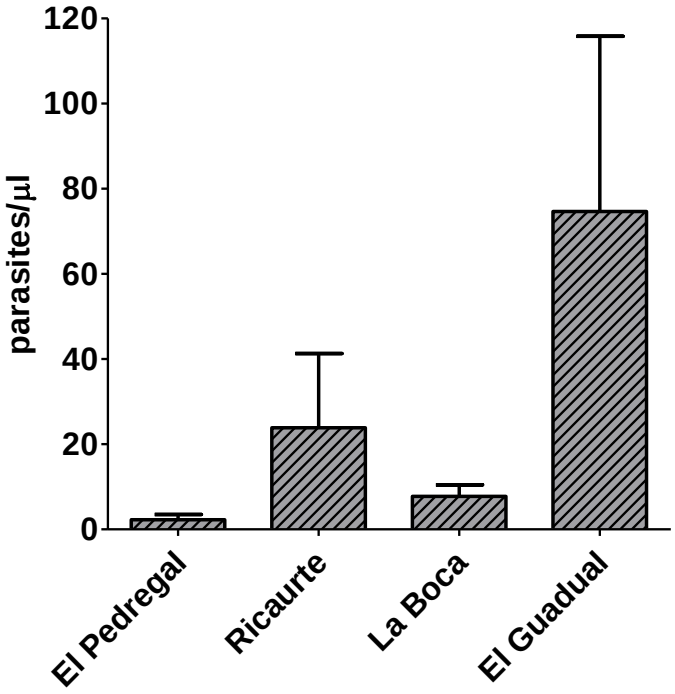
A.


10
8
6
4
2
0
% infection
2
0.7
2.1
0.7
3.1
0.9
8.6
2.2
El Pedregal
Ricaurte
La Boca
El Guadual
P. vivax
P. falciparum

B.


Colombia
Pacific Ocean
ECUADOR
El Pedregal
25%
75%
25%
Ricaurte
22%
75%
La Boca
78%
20%
El Guadual
80%
P. vivax
P. falciparum
N
W
E
S
0
10
20
40
km
Coordinate Systems: GCS WGS 1984
Datum: WGS 1984
Units: Degree
Created by: Andrea López R.

C.



D.

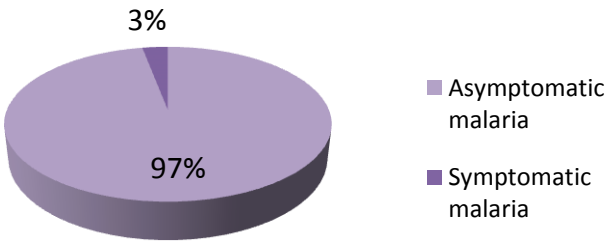
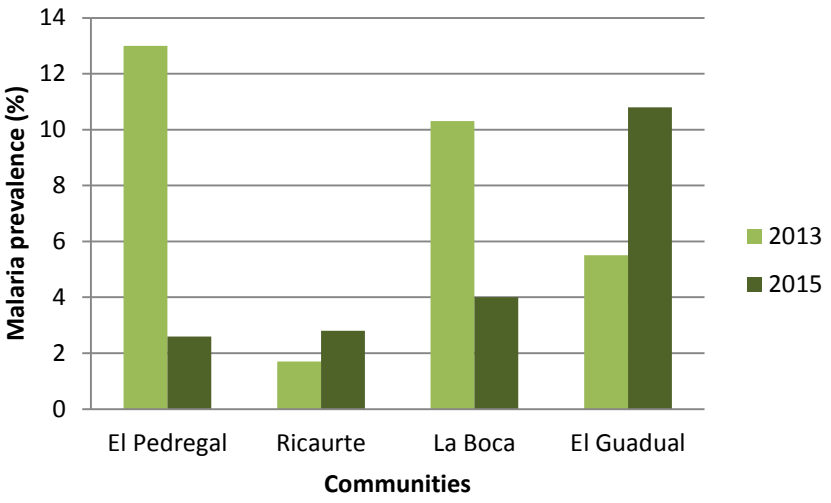


Figure 3. Malaria prevalence in the four study communities of San Lorenzo by qPCR. A) Prevalence of *P. vivax* and *P. falciparum* in the study areas. El Guadual had a higher percentage of cases of both parasites; B) Distribution of *Plasmodium* species as a percentage of positives cases in the communities. *P. vivax* was the most prevalent parasite in all communities; C) Parasitemia levels in the study areas, the mean parasitemia was higher in El Guadual (74.67 parasites/µl), followed by Ricaurte (23.88 parasites/µl); D) Malaria prevalence according symptoms. There was a 97% prevalence of asymptomatic malaria infections.

A.



B.

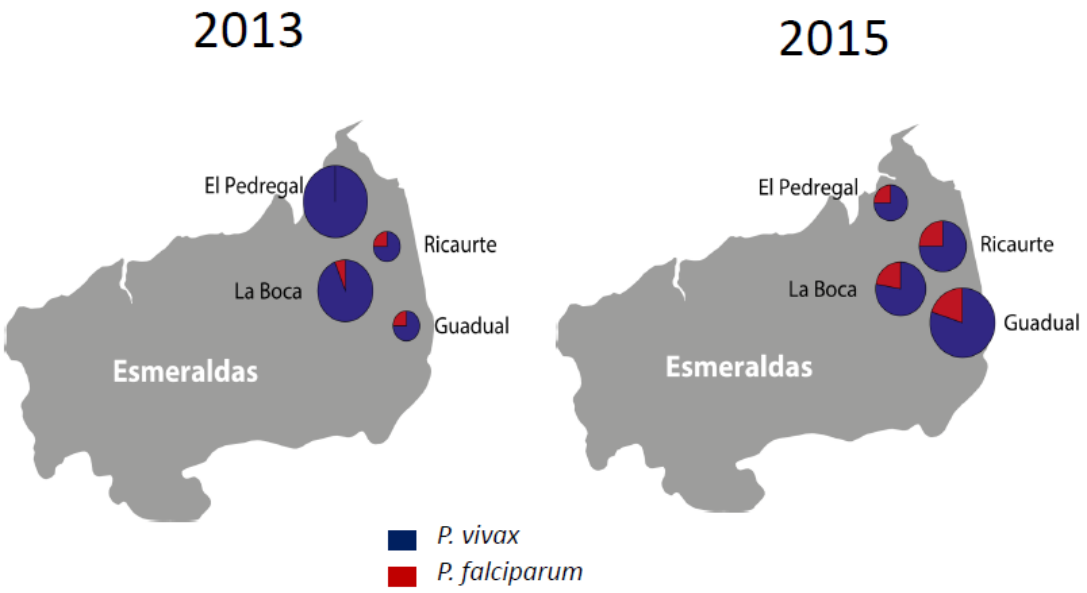


Figure 4. Comparison of malaria prevalence in the study sites in 2013 and 2015. A) Malaria prevalence in 2013 and 2015. There is an 80% and 61.2% prevalence decrease in El Pedregal and La Boca respectively. There is a 64.7% and 96.4% increase of prevalence in Ricaurte and El Guadual respectively; B) Distribution of positive cases by species in the study sites. An increase of *P. falciparum* prevalence (100%) in all communities and an increase of 96.4% in prevalence cases in the highlands region (El Guadual) were observed. The circle size is proportional to the prevalence (Map: CLAIM, 2015).

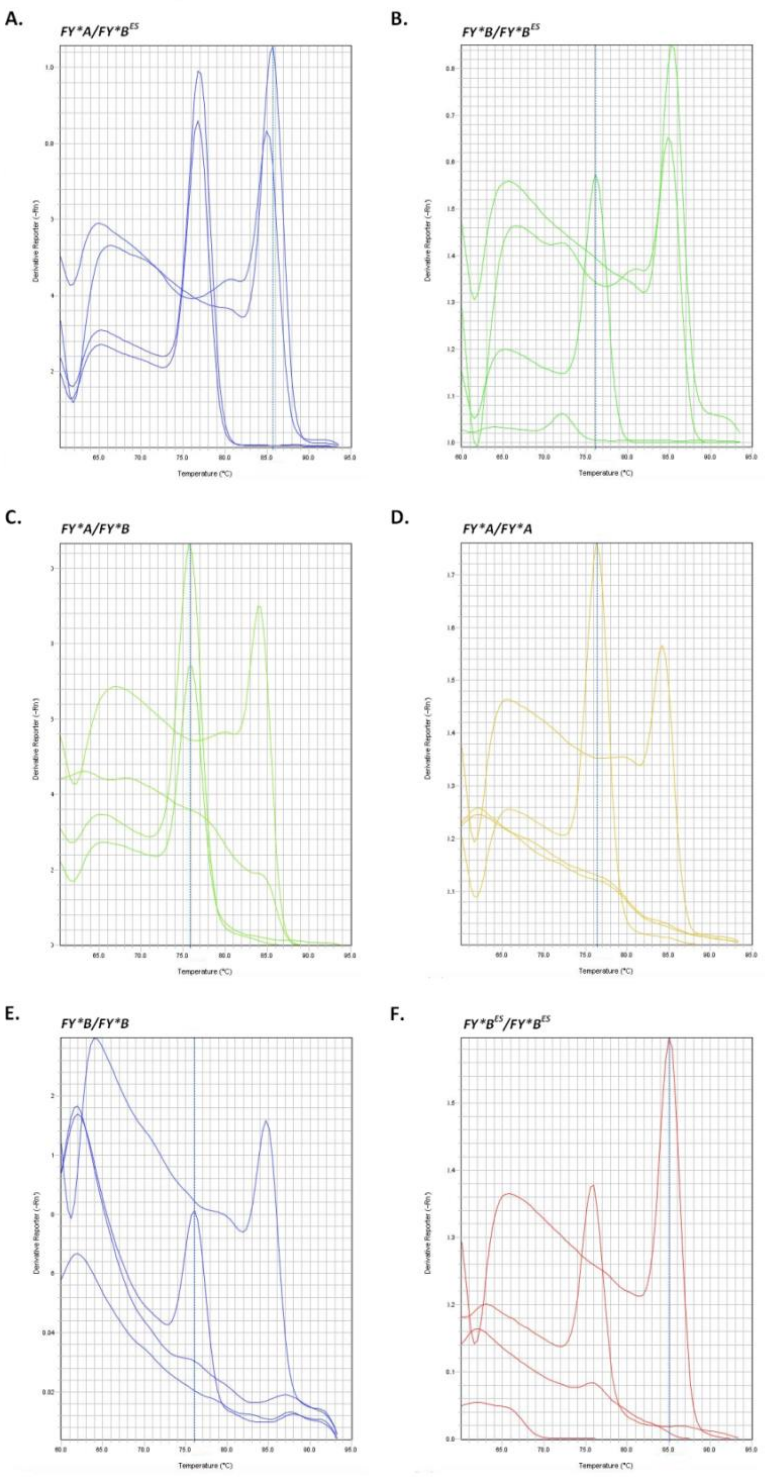
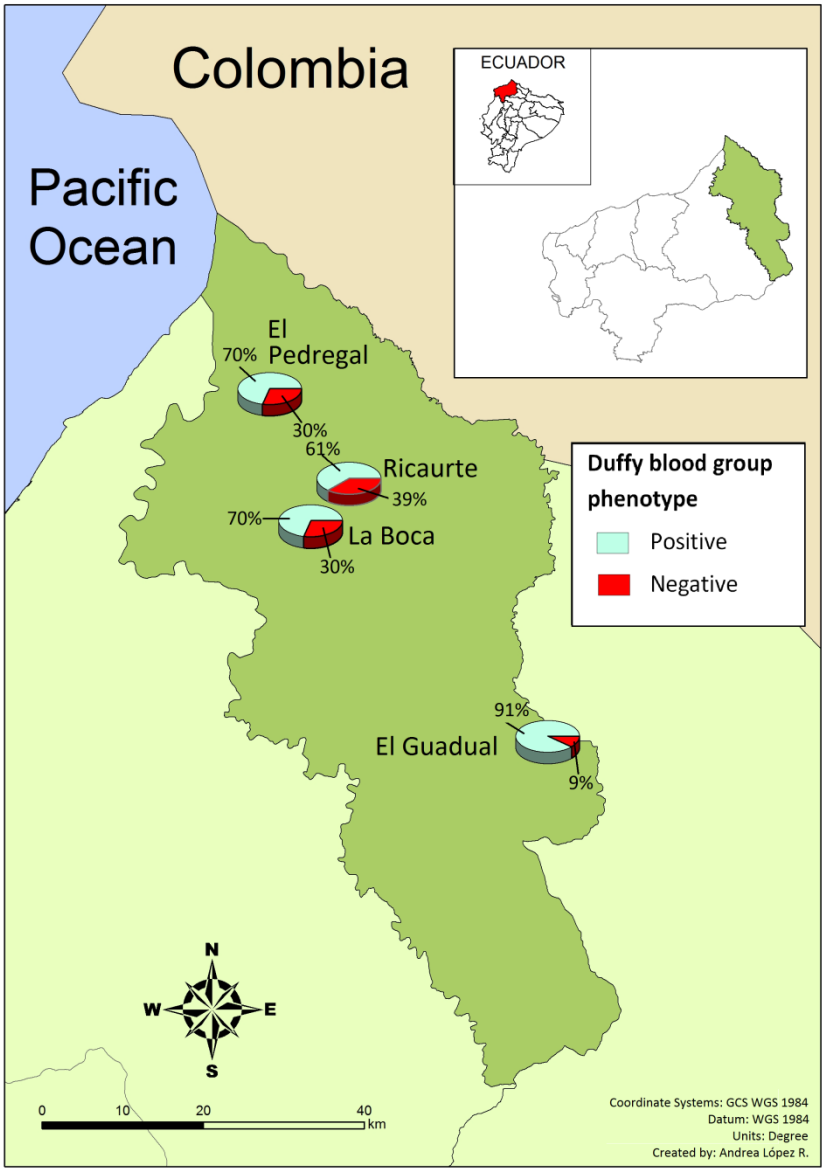


Figure 5. Representative curves of Duffy genotyping by real-time PCR. The graphics show the melting curves obtained using four multiplex reactions. The results were interpreted using the melting temperature of amplified alleles. *FY*A* and *FY*B* alleles have a melting temperature of 77-78°C. The wild type and mutation at position -33 in the GATA box have a melting temperature of 86-88°C. A) Genotype *FY*A/FY*B*^{ES}. Four melting

curves were observed: allele A, allele B, wild type and mutation at position -33; B) Genotype *FY*B/FY*B*^{ES}. Three melting curves were observed: allele B, wild type and mutation at position -33; C) Genotype *FY*A/FY*B*. Three melting curves were observed: allele A, allele B and wild type at position -33; D) Genotype *FY*A/FY*A.* Two melting curves were observed: allele A and wild type at position -33*;* E) Genotype *FY*B/FY*B.* Two melting curves were observed: allele B and wild type at position -33; F) Genotype *FY*B*^{ES}*/FY*B*^{ES}. Two melting curves were observed: allele B and mutation at position -33.

A.


Colombia
Pacific Ocean
ECUADOR
El Pedregal
70%
30%
61%
Ricaurte
39%
70%
La Boca
30%
91%
El Guadual
9%
Duffy blood group phenotype
Positive
Negative
N
W
E
S
0
10
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40
km
Coordinate Systems: GCS WGS 1984
Datum: WGS 1984
Units: Degree
Created by: Andrea López R.

B.

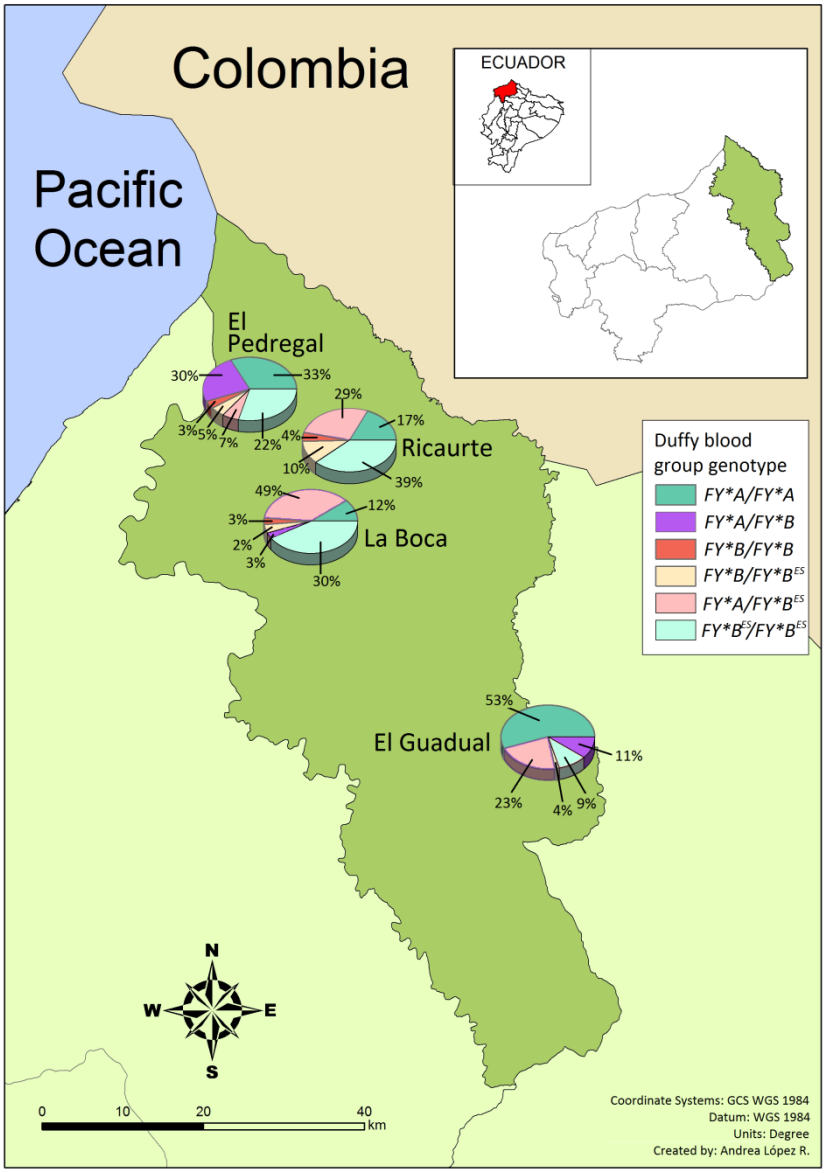

Figure 6. Prevalences of Duffy blood group phenotypes and genotypes. A) Pie graphs showing the distribution of prevalences of Duffy positive and Duffy negative phenotypes in the study communities. We observed an association between the Duffy positive phenotype with El Guadual (91.1%) and an association between Duffy negative and lowland communities (34%) (p=0.000); B) Pie graphs showing distribution of prevalences of Duffy genotypes. The Duffy blood group system is highly heterogeneous. Ricaurte and La Boca populations had a specific genotypic composition where the predominant genotypes were *FY*A/FY*B*^{ES} and *FY*B*^{ES}*/FY*B*^{ES}, which differ from the other two communities where the predominant genotype was *FY*A/FY*A.*

Tables

Table 1. Primers and probes used in this study.

Primer/direction	Sequence	Use
PfCoxIF/sense	5´-CAA CCA ATT GCT TTT GTT TTA G-3´	Detection of *P. falciparum*
PfCoxIR/reverse	5´-ACA GGA GAT AAT GAC ATT AAA GA-3´	Detection of *P. falciparum*
PvCoxIF/sense	5´-CAG CAG AAT TTG GAG GAG-3´	Detection of *P. vivax*
PvCoxIR/reverse	5´-CTA GCA ATA CCA GAT ACT AAA AG-3´	Detection of *P. vivax*
P. falciparum N	/56-FAM/AAG TCC ATC /ZEN/CAG TTC CAC CAC /3IABkFQ/	Detection of *P. falciparum*
P. vivax N	/5HEX/ACA ATG ATA /ZEN/ACA TCT ACT GCA ACA GGA /3IABkFQ/	Detection of *P. vivax*
FAB/sense	5´-CCC TCA TTA GTC CTT GGC TCT TTT-3´	Identification of wild type at position -33 (T)
FGATA/sense	5´-CCCGGGCCCGCCG CCC TCA TTA GTC CTT GGC TCT TTC-3´	Identification of mutation at position -33 (C)
RABGATA/reverse	5´-A GGG GCA TAG GGA TAA GGG ACT-3´	Identification of wild type and mutation at position -33
FY/sense	5´-C TCA AGT CAG CTG GAC TTC GAA GAT-3´	Identification of Fy*A and Fy*B alleles
RYA/reverse	5´-AG CTG CTT CCA GGT TGG CTC-3´	Identification of Fy*A allele
RYB/reverse	5´-CTG CTT CCA GGT TGG CGT-3´	Identification of Fy*B allele

Table 2. Malaria prevalence by qPCR in the study sites.

	Positive cases		***P. vivax***		***P. falciparum***	
Community	**n**	**%**	**n**	**%**	**n**	**%**
El Pedregal (n=151)	4	2.6	3	2	1	0.7
Ricaurte (n=281)	8	2.8	6	2.1	2	0.7
La Boca (n=226)	9	4	7	3.1	2	0.9
El Guadual (n=139)	15	10.8	12	8.6	3	2.2
TOTAL (N=797)	**36**	**4.5**	**28**	**3.5**	**8**	**1**

Table 3. Comparison of malaria prevalence in the study sites in 2013 and 2015.

	2013	**2015**	**% of increase or decrease in prevalence**	**p-value[a]**
Malaria prevalence	7.4%	4.5%	Decrease of 39.2%	**0.013**
El Pedregal	13.0%	2.6%	Decrease of 80%	**0.001**
Ricaurte	1.7%	2.8%	Increase of 64.7%	0.299
La Boca	10.3%	4.0%	Decrease of 61.2%	**0.012**
El Guadual	5.5%	10.8%	Increase of 96.4%	0.150
P. vivax prevalence	7.0%	3.5%	Decrease of 50%	**0.002**
P. falciparum prevalence	0.5%	1.0%	Increase of 100%	0.194
Asymptomatic malaria prevalence	98%	97%	Decrease of 1%	0.676

[a] P-value was determined by χ^2 analysis

Table 4. Duffy blood group genotypes and phenotypes by real-time multiplex allele-specific PCR in four communities of San Lorenzo.

Predicted Phenotype n (%)			**Genotype Result n (%)**		**Allele frequency**	
Duffy positive 224 (70.4)	Fy(a+b-)	183 (57.55)	*FY*A/FY*A*	80 (25.16)	*FY*A*	0.436
			*FY*A/FY*B*^{ES}	103 (32.39)		
	Fy(a-b+)	27 (8.49)	*FY*B/FY*B*	9 (2.83)	*FY*B*	0.079
			*FY*B/FY*B*^{ES}	18 (5.66)		
	Fy(a+b+)	14 (4.40)	*FY*A/FY*B*	14 (4.40)		
Duffy negative 94 (29.6)	Fy(a-b-)	94 (29.56)	*FY*B*^{ES}*/FY*B*^{ES}	94 (29.56)	*FY*B*^{ES}	0.486

Table 5. Prevalence of Duffy phenotypes, genotypes and alleles in lowland communities and El Guadual.

	Lowland communities[a]	El Guadual	p-value[b]
Duffy phenotype (predicted)			
Positives	66%	91.1%	**0.000**
Negative	34%	8.9%	**0.000**
Duffy genotype			
*FY*A/FY*A*	19.1%	53.6%	**0.000**
*FY*A/FY*B*^{ES}	34.4%	23.2%	0.070
*FY*B/FY*B*	3.4%	-	0.171
*FY*B/FY*B*^{ES}	6.1%	3.6%	0.356
*FY*A/FY*B*	3.1%	10.7%	**0.022**
*FY*B*^{ES}*/FY*B*^{ES}	34.0%	8.9%	**0.000**
Alleles			
*FY*A*	56.1%	87.5%	**0.000**
*FY*B*	12.2%	14.3%	0.406
*FY*B*^{ES}	74.4%	35.7%	**0.000**

[a]El Pedregal, Ricaurte and La Boca communities

[b]P-value was determined by χ^2 analysis and the Fisher´s exact test.

Table 6. Predicted Duffy phenotypes in *P. vivax* infected and non-vivax infected individuals in four communities of San Lorenzo.

Place	Duffy phenotype (predicted)		*P. vivax* infected n (%)	Controls[a] n (%)	p-value[b]
El Pedregal	Positive		2 (66.7)	40 (70.2)	0.665
		Fy(a+b-)	2 (66.7)	31 (54.39)	0.576
		Fy(a-b+)	0	5 (8.77)	0.767
		Fy(a+b+)	0	4 (7.02)	0.810
	Negative	Fy(a-b-)	1 (33.3)	17 (29.8)	0.665
Ricaurte	Positive		2 (33.3)	66 (62.3)	0.163
		Fy(a+b-)	1 (16.7)	51 (48.11)	0.140
		Fy(a-b+)	1 (16.7)	14 (13.21)	0.587
		Fy(a+b+)	0	1 (0.94)	0.946
	Negative	Fy(a-b-)	4 (66.7)	40 (37.7)	0.163
La Boca	Positive		6 (85.7)	57 (68.7)	0.320
		Fy(a+b-)	6 (85.7)	49 (59.04)	0.163
		Fy(a-b+)	0	5 (6.02)	0.661
		Fy(a+b+)	0	3 (3.61)	0.782
	Negative	Fy(a-b-)	1 (14.3)	26 (31.3)	0.320
El Guadual	Positive		12 (100)	39 (88.6)	0.284
		Fy(a+b-)	9 (75.0)	34 (77.27)	0.571
		Fy(a-b+)	1 (8.33)	1 (2.27)	0.386
		Fy(a+b+)	2 (16.7)	4 (9.09)	0.381
	Negative	Fy(a-b-)	0 (0.0)	5 (11.4)	0.284
Total	Positive		22 (78.6)	202 (69.7)	0.224
		Fy(a+b-)	18 (64,3)	165 (56,9)	0.292
		Fy(a-b+)	2 (7,1)	25 (8,6)	0.586
		Fy(a+b+)	2 (7,1)	12 (4,1)	0.354
	Negative	Fy(a-b-)	6 (21.4)	88 (30.3)	0.224

[a]*P. falciparum* infected and non-infected individuals

[b]P-value was determined by χ^2 analysis and the Fisher´s exact test.

Table 7. Duffy genotype based on qPCR in *P. vivax* infected and non-vivax infected individuals in four communities of San Lorenzo.

Place	**Duffy genotype**	***P. vivax* infected n (%)**	**Controls[a] n (%)**	**p-value[b]**
El Pedregal	*FY*A/FY*A*	1 (33.3)	19 (33.3)	0.745
	*FY*A/FY*B*^{ES}	1 (33.3)	12 (21.1)	0.526
	*FY*B/FY*B*	0 (0.0)	2 (3.5)	0.902
	*FY*B/FY*B*^{ES}	0 (0.0)	3 (5.3)	0.855
	*FY*A/FY*B*	0 (0.0)	4 (7.0)	0.810
	*FY*B*^{ES}*/FY*B*^{ES}	1 (33.3)	17 (29.8)	0.665
Ricaurte	*FY*A/FY*A*	1 (16.7)	18 (17.0)	0.731
	*FY*A/FY*B*^{ES}	0 (0.0)	33 (31.1)	0.116
	*FY*B/FY*B*	0 (0.0)	4 (3.8)	0.800
	*FY*B/FY*B*^{ES}	1 (16.7)	10 (9.4)	0.470
	*FY*A/FY*B*	0 (0.0)	1 (0.9)	0.946
	*FY*B*^{ES}*/FY*B*^{ES}	4 (66.7)	40 (37.7)	0.163
La Boca	*FY*A/FY*A*	1 (14.3)	10 (12.0)	0.612
	*FY*A/FY*B*^{ES}	5 (71.4)	39 (47.0)	0.199
	*FY*B/FY*B*	0 (0.0)	3 (3.6)	0.782
	*FY*B/FY*B*^{ES}	0 (0.0)	2 (2.4)	0.850
	*FY*A/FY*B*	0 (0.0)	3 (3.6)	0.782
	*FY*B*^{ES}*/FY*B*^{ES}	1 (14.3)	26 (31.3)	0.320
El Guadual	*FY*A/FY*A*	9 (75.0)	21 (47.7)	**0.087**
	*FY*A/FY*B*^{ES}	0 (0.0)	13 (29.5)	**0.027**
	*FY*B/FY*B*	0 (0.0)	0 (0.0)	-
	*FY*B/FY*B*^{ES}	1 (8.3)	1 (2.3)	0.386
	*FY*A/FY*B*	2 (16.7)	4 (9.1)	0.381
	*FY*B*^{ES}*/FY*B*^{ES}	0 (0.0)	5 (11.4)	0.284
Total	*FY*A/FY*A*	12 (42,9)	68 (23,5)	**0.025**
	*FY*A/FY*B*^{ES}	6 (21,4)	97 (33,5)	0.138
	*FY*B/FY*B*	0 (0,0)	9 (3,1)	0.431
	*FY*B/FY*B*^{ES}	2 (7,1)	16 (5,5)	0.484
	*FY*A/FY*B*	2 (7,1)	12 (4,1)	0.354
	*FY*B*^{ES}*/FY*B*^{ES}	6 (21,4)	88 (30,3)	0.224

[a]*P. falciparum* infected and non-infected individuals

[b]P-value was determined by χ^2 analysis and the Fisher´s exact test.

Table 8. Allele frequencies in *P. vivax* infected and non-vivax infected individuals in four communities of San Lorenzo.

Place	**Alleles**	***P. vivax* infecteded (frequency)**	**Controls[a] (frequency)**	**p-value[b]**
El Pedregal	*FY*A*	0.500	0.474	0.65
	*FY*B*	0	0.096	0.646
	$FY*B^{ES}$	0.500	0.430	0.601
Ricaurte	*FY*A*	0.167	0.330	0.130
	*FY*B*	0.083	0.090	0.613
	$FY*B^{ES}$	0.750	0.580	0.620
La Boca	*FY*A*	0.500	0.373	0.214
	*FY*B*	0	0.066	0.509
	$FY*B^{ES}$	0.500	0.560	0.605
El Guadual	*FY*A*	0.833	0.670	0.530
	*FY*B*	0.125	0.057	0.224
	$FY*B^{ES}$	0.042	0.273	**0.024**
Total	*FY*A*	0.571	0.422	0.181
	*FY*B*	0.071	0.079	0.480
	$FY*B^{ES}$	0.357	0.498	**0.033**

[a]*P. falciparum* infected and non-infected individuals

[b]P-value was determined by χ^2 analysis and the Fisher´s exact test.

PLOS Neglected Tropical Diseases

Submission Guidelines

PLOS Neglected Tropical Diseases publishes original research articles of importance to the NTDs community and the wider health community. We will consider manuscripts of any length; we encourage the submission of both substantial full-length bodies of work and shorter manuscripts that report novel findings that might be based on a more limited range of experiments.

The writing style should be concise and accessible, avoiding jargon so that the paper is understandable for readers outside a specialty or those whose first language is not English. Editors will make suggestions for how to achieve this, as well as suggestions for cuts or additions that could be made to the article to strengthen the argument. Our aim is to make the editorial process rigorous and consistent, but not intrusive or overbearing. Authors are encouraged to use their own voice and to decide how best to present their ideas, results, and conclusions.

PLOS Neglected Tropical Diseases is committed to the highest ethical standards in medical research. Accordingly, we ask authors to provide specific information regarding ethical treatment of research participants, patient consent, patient privacy, protocols, authorship, and competing interests. We also ask that reports of certain specific types of studies adhere to generally accepted standards. Our requirements are based on the Uniform Requirements for Manuscripts Submitted to Biomedical Journals, issued by the International Committee for Medical Journal Editors.

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File format Manuscript files can be in the following formats: DOC, DOCX, RTF, or PDF. Microsoft Word documents should not be locked or protected. LaTeX manuscripts must be submitted as PDFs. Read the LaTeX guidelines.

Length Manuscripts can be any length. There are no restrictions on word count, number of figures, or amount of supporting information. We encourage you to present and discuss your findings concisely.

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Footnotes Footnotes are not permitted. If your manuscript contains footnotes, move the information into the main text or the reference list, depending on the content.

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Abbreviations Define abbreviations upon first appearance in the text. Do not use non-standard abbreviations unless they appear at least three times in the text. Keep abbreviations to a minimum.

Reference style PLOS uses "Vancouver" style, as outlined in the ICMJE sample references. See reference formatting examples and additional instructions below.

Equations We recommend using MathType for display and inline equations, as it will provide the most reliable outcome. If this is not possible, Equation Editor is acceptable. Avoid using MathType or Equation Editor to insert single variables (e.g., "$a^2 + b^2 = c^2$"), Greek or other symbols (e.g., β, Δ, or ′ [prime]), or mathematical operators (e.g., x, ≥, or ±) in running text. Wherever possible, insert single symbols as normal text with the correct Unicode (hex) values. Do not use MathType or Equation Editor for only a portion of an

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Nomenclature Use correct and established nomenclature wherever possible.

Units of measurement Use SI units. If you do not use these exclusively, provide the SI value in parentheses after each value. Read more about SI units.

Drugs Provide the Recommended International Non-Proprietary Name (rINN).

Species names Write in italics (e.g., *Homo sapiens*). Write out in full the genus and species, both in the title of the manuscript and at the first mention of an organism in a paper. After first mention, the first letter of the genus name followed by the full species name may be used (e.g., *H. sapiens*).

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Most manuscripts should be organized as follows. Instructions for each element appear below.

- Title
- Authors and Affiliations
- Abstract
- Author Summary
- Introduction
- Methods

- Results
- Discussion
- Acknowledgments
- References
- Supporting information Captions

Uniformity in format facilitates the experience of readers and users of the journal. To provide flexibility, however, the Results and Discussion can be combined into one Results/Discussion section.

Other elements

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Parts of a Submission

Title

Include a full title and a short title for the manuscript.

Title	Length	Guidelines	Examples
Full title	250 characters	Specific, descriptive, concise, and comprehensible to readers outside the field	Impact of Cigarette Smoke Exposure on Innate Immunity: A *Caenorhabditis elegans* Model Solar Drinking Water Disinfection (SODIS) to Reduce Childhood Diarrhoea in Rural Bolivia: A Cluster-Randomized, Controlled Trial
Short title	50 characters	State the topic of the study	Cigarette Smoke Exposure and Innate Immunity SODIS and Childhood Diarrhoea

Titles should be written in title case (all words capitalized except articles, prepositions, and conjunctions). Avoid specialist abbreviations if possible. For clinical trials, systematic reviews, or meta-analyses, the subtitle should include the study design.

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On the title page, write author names in the following order:

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Upload a cover letter as a separate file in the online system.

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If your study addresses an infection that is outside our detailed scope, you must first send a presubmission inquiry indicating why you consider the infection to be a neglected tropical disease.

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Distinct from the scientific abstract, the Author Summary should highlight where the work fits in a broader context of life science knowledge and why these findings are important to an audience that includes both scientists and non-scientists. Ideally aimed to a level of understanding of an undergraduate student, the significance of the work should be presented simply, objectively, and without exaggeration.

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This section should provide enough detail for reproduction of the findings. Protocols for new methods should be included, but well-established protocols may simply be referenced. Detailed methodology or supporting information relevant to the methodology can be published on our web site.

This section should also include a section with descriptions of any statistical methods employed. These should conform to the criteria outlined by the Uniform Requirements, as follows:

Describe statistical methods with enough detail to enable a knowledgeable reader with access to the original data to judge its appropriateness for the study and to verify the reported results. When possible, quantify findings and present them with appropriate indicators of measurement error or uncertainty (such as confidence intervals). Avoid relying solely on statistical hypothesis testing, such as P values, which fail to convey important information about effect size and precision of estimates. References for the design of the study and statistical methods should be to standard works when possible (with pages stated). Define statistical terms, abbreviations, and most symbols. Specify the statistical software package(s) and versions used. Distinguish prespecified from exploratory analyses, including subgroup analyses.

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The Results section should include all relevant positive and negative findings. The section may be divided into subsections, each with a concise subheading. The Results section should be written in past tense.

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Give numeric results not only as derivatives (for example, percentages) but also as the absolute numbers from which the derivatives were calculated, and specify the statistical significance attached to them, if any. Restrict tables and figures to those needed to explain the argument of the paper and to assess supporting data. Use graphs as an alternative to tables with many entries; do not duplicate data in graphs and tables. Avoid nontechnical uses of technical terms in statistics, such as "random" (which implies a randomizing device), "normal," "significant," "correlations," and "sample."

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- How can future research build on these observations and what are the key experiments that must be done?

Acknowledgments

Those who contributed to the work but do not meet our authorship criteria should be listed in the Acknowledgments with a description of the contribution.

Authors are responsible for ensuring that anyone named in the Acknowledgments agrees to be named.

References

Any and all available works can be cited in the reference list. Acceptable sources include:

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A reference management tool, EndNote, offers a current style file that can assist you with the formatting of your references. If you have problems with any reference management program, please contact the source company's technical support.

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Source	Format
Published articles	Hou WR, Hou YL, Wu GF, Song Y, Su XL, Sun B, et al. cDNA, genomic sequence cloning and overexpression of ribosomal protein gene L9 (rpL9) of the giant panda (*Ailuropoda melanoleuca*). Genet Mol Res. 2011;10: 1576-1588. Devaraju P, Gulati R, Antony PT, Mithun CB, Negi VS. Susceptibility to SLE in South Indian Tamils may be influenced by genetic selection pressure on TLR2 and TLR9 genes. Mol Immunol. 2014 Nov 22. pii: S0161-5890(14)00313-7. doi: 10.1016/j.molimm.2014.11.005 *Note: A DOI number for the full-text article is acceptable as an alternative to or in addition to traditional volume and page numbers.*
Accepted, unpublished articles	Same as published articles, but substitute "In press" for page numbers or DOI.
Web sites or online articles	Huynen MMTE, Martens P, Hilderlink HBM. The health impacts of globalisation: a conceptual framework. Global Health. 2005;1: 14. Available: http://www.globalizationandhealth.com/content/1/1/14.
Books	Bates B. Bargaining for life: A social history of tuberculosis. 1st ed. Philadelphia: University of Pennsylvania Press; 1992.
Book chapters	Hansen B. New York City epidemics and history for the public. In: Harden VA, Risse GB, editors. AIDS and the historian. Bethesda: National Institutes of Health; 1991. pp. 21-28.
Deposited articles (preprints, e-prints, or arXiv)	Krick T, Shub DA, Verstraete N, Ferreiro DU, Alonso LG, Shub M, et al. Amino acid metabolism conflicts with protein diversity; 1991. Preprint. Available: arXiv:1403.3301v1. Accessed 17 March 2014.
Published media (print or online newspapers and magazine	Fountain H. For Already Vulnerable Penguins, Study Finds Climate Change Is Another Danger. The New York Times. 29 Jan 2014. Available: http://www.nytimes.com/2014/01/30/science/earth/climate-change-taking-toll-on-penguins-study-finds.html. Accessed 17 March

Source	Format
articles)	2014.
New media (blogs, web sites, or other written works)	Allen L. Announcing PLOS Blogs. 2010 Sep 1 [cited 17 March 2014]. In: PLOS Blogs [Internet]. San Francisco: PLOS 2006 - . [about 2 screens]. Available: http://blogs.plos.org/plos/2010/09/announcing-plos-blogs/.
Masters' theses or doctoral dissertations	Wells A. Exploring the development of the independent, electronic, scholarly journal. M.Sc. Thesis, The University of Sheffield. 1999. Available: http://cumincad.scix.net/cgi-bin/works/Show?2e09
Databases and repositories (Figshare, arXiv)	Roberts SB. QPX Genome Browser Feature Tracks; 2013. Database: figshare [Internet]. Accessed: http://figshare.com/articles/QPX_Genome_Browser_Feature_Tracks/701214.
Multimedia (videos, movies, or TV shows)	Hitchcock A, producer and director. Rear Window [Film]; 1954. Los Angeles: MGM.

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S1 Text. Title is strongly recommended. Legend is optional.

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Competing interests

The corresponding author is asked at submission to declare, on behalf of all authors, whether there are any financial, personal, or professional interests that could be construed to have influenced the work.

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Clinical trials

We follow the World Health Organization’s (WHO) definition of a clinical trial:

A clinical trial is any research study that prospectively assigns human participants or groups of humans to one or more health-related interventions to evaluate the effects on health outcomes [...] Interventions include but are not restricted to drugs, cells and other biological products, surgical procedures, radiologic procedures, devices, behavioural treatments, process-of-care changes, preventive care, etc.

PLOS Neglected Tropical Diseases requires that all trials be registered and, as of August 13, 2013, supports the position of the AllTrials.net Initiative that trials that are registered after the trial commences or retrospectively will be considered (see the blog post for more details). For all trials, authors are asked to provide the trial registration information and to register their trial in an approved registry (the WHO's list of approved registries is listed here). For trials that were registered after the trial began or retrospectively, authors are asked to provide the following information:

- The trial registration information (or indicate that registration is in process)
- The reason for late registration, explained within the Methods section
- A statement in which all authors affirm that any trials on the same or a related drug or intervention they're involved in are registered, and provide (either as part of the statement or in the supplementary information) links to the published versions of the trials or the registration numbers. This statement will be published in the Methods section.

The editors reserve the right to inform authors' institutions or ethics committees about unregistered trials that have been carried out. Authors will also be asked to submit an accurate summary of the trial's results to the relevant registry (if there is such a mechanism) within a year of study completion or at the time of publication, whichever is the earliest.

Authors of trials must adhere to the CONSORT reporting guidelines appropriate to their trial design. Please check the CONSORT statement web site for information on the appropriate guidelines for specific trial types. Before the paper can undergo peer review, authors must: 1) provide in the manuscript the trial registry, trial registration number, and IRB, and 2) provide a copy of the trial protocol (or a link to an open access version of the protocol) and a completed CONSORT checklist as supporting files (these documents will also be published alongside the paper, if accepted). The CONSORT flow diagram must be included as Figure 1. Any deviation from the trial protocol must be explained in the paper. Authors must explicitly discuss informed consent in their paper, and PLOS reserves the right to request a copy of the patient consent form. Information on statistical methods or participants beyond what is indicated in the CONSORT statement should be reported in the Methods section.

PLOS supports the public disclosure of all clinical trial results, as mandated, for example, by the FDA Amendments Act, 2007. For trials in registries that permit posting of trial results, PLOS Neglected Tropical Diseases requires that an accurate summary of the trial's results be submitted to the relevant registry (if there is such a mechanism) within a year of study completion or at the time of publication, whichever is the earliest.

Systematic reviews and meta-analyses

Reports of systematic reviews and meta-analyses must adhere to the PRISMA Statement or alternative guidelines appropriate to the study design, and include the completed checklist and flow diagram to accompany the main text. Authors must complete the appropriate reporting checklist not only with page references, but also with sufficient text excerpted from the manuscript to explain how they accomplished all applicable items.

Abstracts should follow PRISMA for Abstracts, using the PLOS abstract format. Authors must also state within the Methods section of their paper whether a protocol exists for their systematic review, and if so, provide a copy of the protocol as supporting information. The journal supports the prospective registration of systematic reviews. Authors whose systematic review was prospectively registered (e.g., in a registry such as PROSPERO) should provide the registry number in their abstract. Registry details and protocols will be made available to editors and reviewers, and included with the paper if the report is ultimately published.

Diagnostic studies

Reports of studies of diagnostic accuracy must adhere to the STARD requirements or alternative guidelines appropriate to the study design (see the EQUATOR web site) and include a completed checklist as supporting information. Authors must complete the appropriate reporting checklist not only with page references, but also with sufficient text excerpted from the manuscript to explain how they addressed all applicable items.

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For observational studies, including case control, cohort, and cross-sectional studies, authors must adhere to the STROBE Statement or alternative guidelines appropriate to the study design (see the EQUATOR web site) and include a completed checklist as supporting information. Authors must complete the appropriate reporting checklist not only with page references, but also with sufficient text excerpted from the manuscript to explain how they addressed all applicable items.

For observational studies, authors are required to clearly specify (a) What specific hypotheses the researchers intended to test, and the analytical methods by which they planned to test them; (b) What analyses they actually performed; and (c) When reported

analyses differ from those that were planned, authors must provide transparent explanations for differences that affect the reliability of the study's results.

If a prospective analysis plan (from the study's funding proposal, IRB or other ethics committee submission, study protocol, or other planning document written before analyzing the data) was used in designing an observational study, authors must include the relevant prospectively written document with the manuscript submission for access by editors and reviewers and eventual publication alongside the accepted paper. If no prospectively written document exists, authors should explain how and when they determined the analyses being reported.

Microarray experiments

Reports of microarray experiments must conform to the MIAME guidelines, and the data from the experiments must be deposited in a publicly accessible database.

Other Article Types

If you are submitting content other than a research article, read the guidelines for other article types.

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DECLARACIÓN Y AUTORIZACIÓN

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Quito, 26 de noviembre de 2015

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